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Poly-β-hydroxybutyrate (PHB) accumulating *Bacillus* spp. improve the survival, growth and robustness of *Penaeus monodon* (Fabricius, 1798) postlarvae

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ABSTRACT

Low larval survival resulting from suboptimal culture conditions and luminous vibriosis poses a major problem for the larviculture of penaeid shrimp. In this study, a poly-β-hydroxybutyrate (PHB) accumulating mixed bacterial culture (mBC; 48.5% PHB on cell dry weight) and two PHB accumulating bacterial isolates, *Bacillus* sp. JL47 (54.7% PHB on cell dry weight) and *Bacillus* sp. JL1 (45.5% PHB on cell dry weight), were obtained from a Philippine shrimp culture pond and investigated for their capacity to improve growth, survival and robustness of *Penaeus monodon* postlarvae (PL). Shrimp PL1 and shrimp PL30 were provided with the PHB containing bacterial cultures in the feed for 30 days followed by, respectively, a challenge with pathogenic *Vibrio campbellii* and exposure to a lethal dose of ammonia. Prior to the pathogenic challenge or ammonia stress, growth and survival were higher for shrimp receiving the PHB accumulating bacteria as compared to shrimp receiving diets without bacterial additions. After exposure to the pathogenic challenge the shrimp fed PHB accumulating bacteria showed a higher survival as compared to non-treated shrimp, suggesting an increase in robustness for the shrimp. Similar effects were observed when shrimp PL30 were provided with the PHB accumulating bacterial cultures during a challenge with pathogenic *V. campbellii* through the water. The survival of shrimp exposed to lethal ammonia stress showed no significant difference between PHB accumulating bacteria-fed shrimp and non-PHB treated shrimp. The data illustrate that bacilli capable of accumulating PHB can provide beneficial effects to *P. monodon* post-larvae during culture in terms of growth performance, survival and resistance against pathogenic infection and ammonia stress. Further investigations are required to verify the PHB effect of the bacterial cultures on the shrimp.

1. Introduction

Outbreaks of luminescent vibriosis are one of the major constraints in the culture of giant tiger prawn, *Penaeus monodon*. This bacterial disease significantly affects the early life stages of the shrimp resulting in major economic losses (Karunasagar et al., 1994; Lavilla-Pitogo et al., 1990). The use of antibiotics as prophylactic agents against bacterial diseases has been proven unsustainable and ineffective due to the development of antibiotic resistance in pathogens (Cabello, 2006). Therefore, several alternative strategies have been proposed to address this problem as described in the review of Defoirdt et al. (2007a). One of

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them is the application of the bacterial storage compound poly-β-hydroxybutyrate (PHB). PHB is a compound accumulated as a cellular energy and carbon reserve by a large variety of bacteria that include the genera *Alcaligenes, Pseudomonas*, *Rhizobium* and *Bacillus* (Anderson and Dawes, 1990; Jiang et al., 2008; Kaynar and Beyatli, 2009; Paganelli et al., 2011; Rebah et al., 2009; Wang et al., 2012). It is deposited intracellularly in amorphous state in inclusions in the cytoplasm (Amor et al., 1991; Barnard and Sanders, 1989), and inclusion levels are depending on the nutritional (i.e. carbon source, C/N ratio, etc.) and the environmental conditions (i.e. pH, oxygen, etc.) during growth (Borah et al., 2002; Du et al., 2000).

Originally, the interest for PHB was based on its medical and industrial application possibilities (Anderson and Dawes, 1990; Chen and Wu, 2005), and it was not until recently that PHB was identified as a potential bio-control agent for aquaculture (Defoirdt et al., 2007b). It was previously shown that the addition of PHB particles in starved *Artemia* resulted in a prolonged survival of the animal suggesting that PHB particles are (at least partially) degraded in the gut and are used by the animal as an energy source during stressful conditions (Defoirdt et al., 2007b). By now, the use of PHB as a biocontrol agent for crustacean culture has been tested and found promising to control vibriosis (Defoirdt et al., 2007b; Nhan et al., 2010; Sui et al., 2012). In these studies, PHB has been applied in a powdered, crystalline form (i.e. extracted from a bacterial cell). The efficiency of the PHB strategy, however, has been shown to be considerably higher when PHB is supplied in amorphous state (i.e. still contained in a bacterial cell). Indeed, an earlier study has shown that PHB containing bacteria may be more effective than PHB powder to protect *Artemia franciscana* from pathogenic *Vibrio campbellii* infection (Halet et al., 2007). The challenge now lies with finding appropriate bacterial cultures capable of accumulating PHB for application in aquaculture settings. In the genus *Bacillus*, PHB is used as an energy source to fuel the sporulation process (Valappil et al., 2007). PHB is accumulated during or shortly after logarithmic growth with a maximum just prior to the formation of spores. During sporulation, PHB is degraded to fuel the process. Bacilli have been shown to accumulate PHB in the range of 11% up to as high as 79% on cell dry weight (CDW) under optimized conditions (Singh et al., 2009; Yüksekdağ et al., 2003). Spore-forming *Bacilli* are commonly found in the gut of fish and shrimp, and *Bacillus megaterium* and *Bacillus pasteurii* originating from the intestinal environment of various fish species have been shown to accumulate PHB up to 23% on CDW (Kaynar and Beyatli, 2009).

Bacillus spp. are generally considered to be interesting probiotic bacteria for aquaculture purposes (Irianto and Austin, 2002). Aside from their various beneficial probiotic effects, their ability to produce spores make them more advantageous over other probiotic species in terms of long term storage and ease of application (Hong et al., 2005). Therefore, using *Bacillus* species with high PHB accumulating capacity could be an interesting approach to apply in aquaculture, while the possibly combined activity of intracellular PHB and the probiotic activity of *Bacillus* species is an interesting feature to explore. In this study, PHB accumulating *Bacillus* spp. were therefore isolated and tested in *P. monodon* postlarvae (PL) culture. Their effects on survival, growth and robustness against stress of the shrimp PL during culture were determined. This study is the first attempt of using PHB accumulating *Bacillus* spp. in larviculture.

2. Materials and methods

2.1. PHB accumulating bacterial cultures

A sediment sample was collected from a shrimp pond in Bacolod (Philippines) and pasteurized (80 °C, 20 min) to obtain a mixed bacterial culture (mBC) consisting of spore-forming *Bacillus* spp. From this mBC, several isolates were purified using the streak plate procedure on Luria Bertani (LB) agar (tryptone (Himedia, India; 10 g L^{-1}), yeast extract (Himedia, India; 5 g L^{-1}) and NaCl (Sigma-Aldrich, Singapore; 20 g L^{-1})). The two isolates with the highest PHB accumulation as assessed by Sudan Black staining of the colonies (Hartman, 1940) and a PHB assay (Law and Slepecky, 1961) were selected, designated as isolate JL1 and isolate JL47 and kept at −80 °C in 20% glycerol as stock cultures.

2.2. Identification of the isolates by 16s rRNA gene sequencing

A single colony of each isolate was picked using a sterilized toothpick and suspended in 0.5 mL sterile saline water and then centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was suspended in 0.5 mL of InstaGene Matrix (Bio-Rad USA) and incubated for 30 min. at 56 °C and then heated at 100 °C for 10 min. After heating, the supernatant was used for PCR. A 1 μL aliquot of the template DNA was added in 20 μL PCR reaction solution. PCR targeting a ca. 1,400 bp fragment of the 16S rRNA gene of the isolates was performed with a PTC-225 Peltier Thermal Cycler (MJ Research, USA) using the universal primer pair 27F and 1492R for bacteria (Lane, 1991) and the program was set at 94 °C for 45 s (denaturation), 55 °C for 60 s (annealing) and 72 °C for 60 s (extension) for 35 cycles. Purification of the amplified product was done using a Montage PCR Clean up kit (Millipore). Sequencing reactions were performed using primer pair 518F and 800R (Anzai et al., 1997) and a BigDye Terminator Cycle Sequencing kit (Applied BioSystems, USA) and the BigDye XTerminator Purification kit (Applied BioSystems, USA) was used for purification. Sequencing was performed on an Applied BioSystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) at Macrogen, Seoul, Korea. The sequences of the isolates were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank) under the accession numbers KJ496326 and KJ496325 for isolate JL1 and isolate JL47, respectively. Homology searches were completed with the BLAST server of the National Center for Biotechnology Information for comparison of the nucleotide query sequence against a nucleotide sequence database (blastn). The results of the homology searches are presented in Table 1.

Table 1
Results of the homology search for the isolate nucleotide query sequences using the NCBI BLASTN search tool.

Isolate (GenBank accession no.)	Closest match (GenBank accession no.)	Identity similarity (%)
JL1 (KJ496326)	*Bacillus anthracis* Y43 (KF730755.1)	99
	Bacillus cereus AVP12 (KF527826.1)	99
	Bacillus thuringiensis GS1 (FJ462697.1)	99
JL47 (KJ496325)	*Bacillus cereus* SBTBC-008 (KF601957.1)	99
	Bacillus thuringiensis AHBR13 (KF241526.1)	99
	Bacillus anthracis JN22 (KF150341.1)	99

2.3. Feed preparation

The bacterial stock cultures were activated in LB medium for 16 h and subsequently inoculated at 1% (v/v) in LB added with 20 g L^{-1} glucose (Sigma-Aldrich, Singapore). The cultures were grown at 30 °C and 100 rpm agitation for 72 h. After culture, the bacterial cells were harvested by centrifugation at 5,000 rpm for 5 min, discarding the supernatant and washing with sterile saline (8.5 g L^{-1} NaCl) (repeated 3 times).

A formulated shrimp diet containing 40.5% crude protein and 11.5% crude fat was prepared according to Catacutan (1991). During preparation, the shrimp feed was added with either freshly grown JL1, JL47 or mBC at 4 g wet bacterial weight kg^{-1} feed by spraying the bacterial suspensions gradually into the feeds ensuring a homogenous distribution. A final coating of the feed with cod liver oil (CLO) was used to minimize the leaching of bacteria. The control diet without bacteria was also CLO coated. To avoid excess amounts of lipids in the feed, the fraction of CLO used in the final coating of the bacteria was omitted from the formulation during feed preparation. Feeds sprayed with bacteria and CLO were dried at 30 °C for 1 h and kept at −20 °C until needed.

2.4. Measurement of PHB in JL1, JL47 and mBC

At 24, 48 and 72 h of culturing JL1, JL47 and mBC, 3 mL aliquots were sampled and frozen immediately at −80 °C until measurement of PHB. PHB was quantified spectrophotometrically following the procedure of Law and Slepecky (1961) with a few modifications. In brief, samples were harvested as described above and finally centrifuged at 13,000 rpm for 20 min in dried, pre-weighed 2 mL eppendorf tubes with safe-lock. The bacterial pellets were oven dried at 110 °C until constant weight. The dried bacterial pellets were lysed in 1.5 mL sodium hypochlorite (5.25% active chlorine), vortexed and heated in a water bath for 10 min at 60 °C. The samples were centrifuged at 13,000 rpm for 20 min and sodium hypochlorite was carefully removed. The remaining pellets were washed with distilled water, vortexed and centrifuged at 13,000 rpm for 20 min. The supernatant was carefully removed and equal volumes of ethanol and acetone (750 μL) were added. The samples were vortexed, centrifuged at 13,000 rpm for 20 min and the supernatant was carefully removed. The remaining samples were dried in a vacuum desiccator. The dried samples were then added with 2 mL chloroform (98%) and left overnight in a shaker. Aliquots of the chloroform samples (500 μL) were transferred into clean glass vials and the chloroform was evaporated in a vacuum dessicator. The remaining PHB pellet was added with 4 mL sulfuric acid (98%) and heated at 100 °C for 20 min in a water bath to convert PHB into crotonic acid. Samples were cooled to room temperature and two-fold diluted with 0.014 M sulfuric acid. The crotonic acid was measured using a spectrophotometer (UV mini 1240-Shimadzu™) at 235 nm wavelength. PHB concentrations in the diluted aliquots were determined by a standard curve for the optical density (OD) in function of PHB concentration constructed by using crystalline PHB (Sigma-Aldrich, Singapore). The PHB content in the bacterial cells was then calculated by the formula:

$$\text{PHB in bacterial cells (\% dry weight)} = \frac{(\text{conc of PHB in the aliquots(mg PHB/mL)} \times 2 \times 4\ \text{mL}/0.5\ \text{mL} \times 2\ \text{mL})}{(\text{weight bacterial pellet (mg cell dry weight)})} \times 100.$$

2.5. In vivo tests

2.5.1. Experiment 1: Effects of PHB accumulating bacterial cultures on P. monodon PL1

2.5.1.1. Survival. *P. monodon* postlarvae (PL1) were obtained from the Tigbauan Main Station shrimp hatchery of the Southeast Asian Fisheries Development Center—Aquaculture Department, Tigbauan, Iloilo, Philippines and acclimatized to the experimental conditions for a week by stocking the animals in 60 L oval tanks (60 cm length × 40 cm width × 25 cm height) at 20 shrimp L^{-1} with moderate aeration. Temperature ranged from 28 to 30 °C. On a daily basis, the tanks were siphoned and half of the water was replaced with filtered and UV treated seawater. At the start of the experiment, the shrimp from the holding tanks were restocked in 60 L oval tanks at a density of 1 shrimp L^{-1} seawater. During the experimental period of 30 days, the treatments consisted of the formulated shrimp diet added with either JL1, JL47 or mBC ($n = 4$ per treatment). Formulated diet without PHB-accumulating bacteria was used as the control treatment ($n = 4$, but 4 extra tanks were kept with

shrimp under the experimental conditions of the control treatment to provide enough larvae for the challenge test; see next paragraph). Shrimps were fed to satiation and excess feed was siphoned out every morning before feeding. Filtered and UV treated seawater was used for a daily water change of 50%. Survival was recorded at the end of the experiment.

2.5.1.2. Vibrio campbellii LMG 21363 challenge test. After culturing the PL1 for 30 days with the experimental diets, 30 shrimp from each treatment replicate were transferred to a 60 L tank (60 cm length × 40 cm width × 25 cm height). The shrimp were challenged for 24 h with pathogenic *V. campbellii* LMG 21363 at approximately 10^6 colony forming units (CFU) per mL of rearing water in combination with a sub-lethal dose of ammonium chloride (75 mg L^{-1}). *V. campbellii* was previously grown for 16 h in LB medium. Shrimp from the control treatment that were challenged acted as positive controls. Shrimp that were reared in the 4 tanks that were kept in parallel to the control treatment were not challenged and acted as negative controls. After 24 h of challenge, the water containing the pathogen and ammonium chloride was replaced with filtered and UV treated seawater and the mortality of the shrimp was determined at 7 and 15 days post challenge. During the post-challenge monitoring period, shrimp were fed to satiation using the formulated control diet without PHB accumulating bacteria and excess feed was siphoned out every morning before feeding. Filtered and UV treated seawater was used for a daily water change of 50%.

2.5.2. Experiment 2: Effects of PHB accumulating bacterial cultures on P. monodon PL30

2.5.2.1. Growth and survival. P. monodon postlarvae (PL30) were obtained and acclimatized as described for experiment 1. At the start of the experiment, the shrimp from the holding tanks were restocked in 60 L oval tanks (60 cm length × 40 cm width × 25 cm height) at a density of 1 shrimp L^{-1} seawater. The same feeding treatments and rearing procedures from experiment 1 were applied for 30 days ($n = 4$ per treatment). The average body weight (ABW) and average body length (ABL) of the shrimp were determined at the beginning and at the end of the experiment. Survival was recorded at the end of the experiment.

2.5.2.2. Ammonia stress test. After culturing the PL30 for 30 days with the experimental diets, 15 shrimp from 3 replicate tanks per treatment were transferred to 5 L plastic containers (25 cm length × 20 cm width × 10 cm height). A lethal dose of ammonium chloride of about 150 mg L^{-1} was added in each container and survival of the shrimp was determined after 24 h and 48 h of exposure. The ammonium dose was the LD_{50} concentration for shrimp of that age in seawater as determined prior to the experiment. The concentration of non-ionized ammonia (NH_3) at a pH value of 7.8 in the seawater was estimated according to Armstrong et al. (1978) and was about 1.78 mg L^{-1} ammonia. Moderate aeration was provided and no feed was supplied during the 48 h exposure period.

2.5.3. Experiment 3: Effects of PHB accumulating bacterial cultures on P. monodon PL30 challenged daily with Vibrio campbellii LMG 21363

P. monodon postlarvae (PL30) were obtained and acclimatized as described for experiment 1. At the start of the experiment, the shrimp from the holding tanks were restocked in 13.3 L round containers (13 cm radius; 25 cm height) at a density of 2 shrimp L^{-1} seawater ($n = 4$ per treatment). The rearing water was added daily with either JL1, JL47 or mBC at 10^6 CFU mL^{-1} harvested at 48 h of culture. The shrimp were challenged by daily addition of freshly grown *V. campbellii* LMG 21363 at 10^6 CFU mL^{-1} to the rearing water. Shrimp in rearing water without addition of PHB accumulating bacteria or pathogenic bacteria made up the negative control treatment ($n = 4$), while shrimp in rearing water without addition of PHB accumulating bacteria but with addition of pathogenic bacteria made up the positive control treatment ($n = 4$). All shrimp were fed daily with formulated shrimp diet without PHB accumulating bacteria and excess feed was siphoned out every morning prior to feeding. On a daily basis, 90% of the tank water volume was replaced. All bacteria were added after all water management was conducted. Survival of the shrimp was determined at 10 days, 20 days, and 30 days of culture.

2.6. Statistical analysis

All values represent means ± standard error of the mean (SEM). The data on growth and survival were analyzed using one-way ANOVA followed by a post hoc Duncan Multiple Range test for assessment of significant differences between treatments at a 5% significance level ($P < 0.05$) using SPSS 16.0 software. Percentage survival was subjected to arcsine transformation prior to statistical analysis. The Pearson product moment correlation coefficient (r^2) and the Pearson two-tailed P value were determined using GraphPad Prism version 5.03 for Windows, GraphPad Software.

3. Results

3.1. Accumulation of PHB

All PHB accumulating cultures used in this study attained their highest PHB accumulation at 48 h of culture (Fig. 1). In specific, isolate JL47 attained the highest accumulation of PHB (54.7% on CDW) followed by mBC (48.5% on CDW) and isolate JL1 (45.5% on CDW). Based on these values, the PHB doses in the feed could be calculated as 0.41 g PHB kg^{-1} feed, 0.36 g PHB kg^{-1} feed, and 0.34 g PHB kg^{-1} feed for the JL47, mBC and JL1 treatment, respectively.

3.2. Growth and survival of P. monodon postlarvae

The survival of the PL1 and PL30 in experiments 1 and 2, respectively, showed similar trends (Fig. 2). A higher survival was observed for shrimp receiving diets

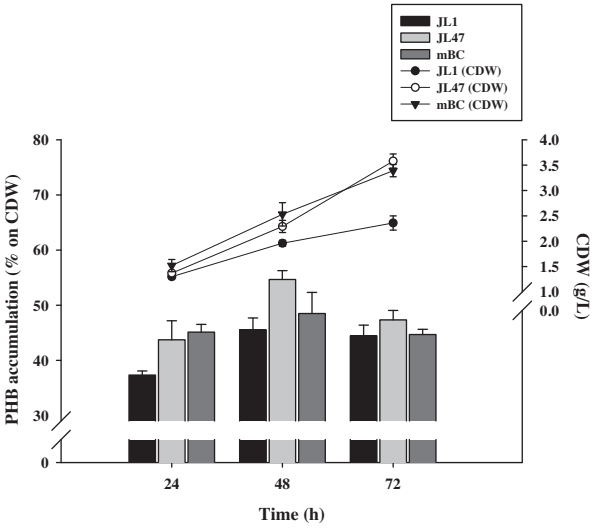

Fig. 1. PHB accumulation and cell dry weight (CDW) concentration of the PHB accumulating bacterial cultures grown in LB + 2% (w/v) glucose.

containing PHB accumulating bacteria as compared to shrimp fed the control diet. In the PL1 experiment, the survival of the JL47 fed shrimp was significantly higher than that of the control fed shrimp. Survival of JL1 fed and mBC fed shrimp was not significantly higher than of control shrimp. In experiment 2, the survival of PL30 fed with JL47 bacteria or JL1 bacteria was significantly higher than that of the control fed PL30. The survival of mBC-fed shrimp was not significantly higher as compared to the survival of the control shrimp.

The ABW and ABL of the PL30 in experiment 2 after 30 days of culture was significantly higher for the JL47 fed shrimp as compared to the control shrimp (Fig. 3). The JL1 and mBC fed shrimp did not show a significantly higher ABW than the control fed shrimp. The ABL of mBC fed shrimp, however, was significantly higher than that of the control shrimp but not significantly different from that of JL1 and JL47 fed shrimps.

3.3. Challenge tests

In the pathogen challenge test from experiment 1, there seemed to be a trend of higher survival for the shrimp fed PHB accumulating bacteria than for the challenged control shrimp after 7 days post-challenge. However, the differences were not significant (Fig. 4A). At 15 days post-challenge, the same trend was observed but now the JL47 fed shrimp survived significantly better than the challenged control shrimp. The non-challenged control attained a higher survival than all other treatments at day 7 and day 15 post-challenge, and the difference was only non-significant when compared to the JL47 and mBC fed shrimp at 7 days post-challenge.

In the pathogen challenge test from experiment 3, similar survival trends were observed as in the challenge test from experiment 1 (Fig. 4B). The survival after 30 days of culture was significantly higher for the shrimp supplied of PHB accumulating JL47 in the rearing water as compared to the positive control shrimp, while this was not the case for the shrimp from the JL1 and mBC treatments. The survival of shrimp supplied of JL47 was comparable to and not significantly different from the non-challenged negative control shrimp.

3.4. Ammonia stress test

The survival of the shrimp after 24 h and 48 h of ammonia challenge (experiment 2) was not significantly different between treatments (Fig. 5). The lowest survival, however, was consistently observed in the challenged positive control indicating a trend of higher survival in case the shrimp were supplied of PHB accumulating bacteria in the diet.

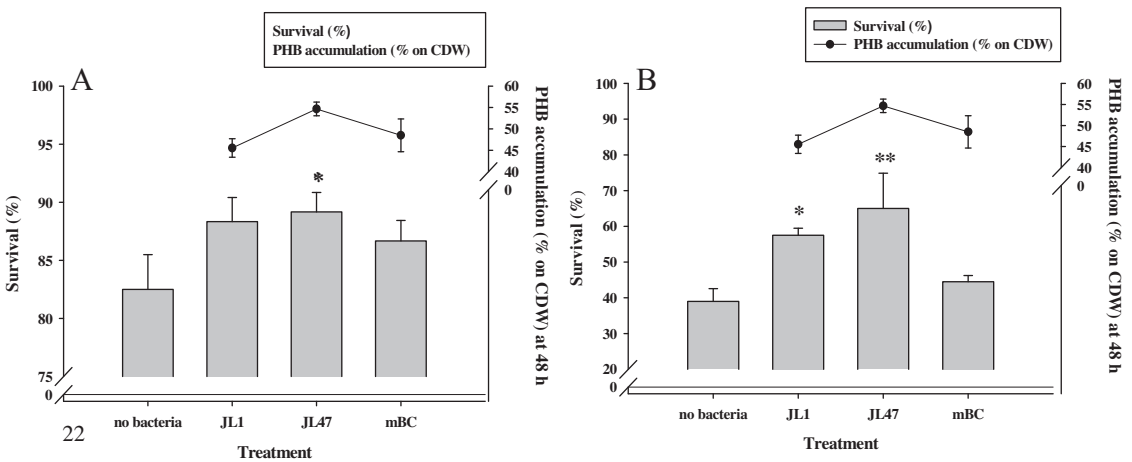

Fig. 2. Survival of shrimp PL1 in experiment 1 (A) and survival of shrimp PL30 in experiment 2 (B) after 30 days of feeding with shrimp formulated diet added with and without different PHB-accumulating bacterial cultures. The PHB content of the bacterial cultures added to the feed is presented. Bars represent means ± SEM ($n = 4$). Asterisks (* $P < 0.05$; ** $P < 0.01$) indicate a significantly different survival as compared to the control (no bacteria).

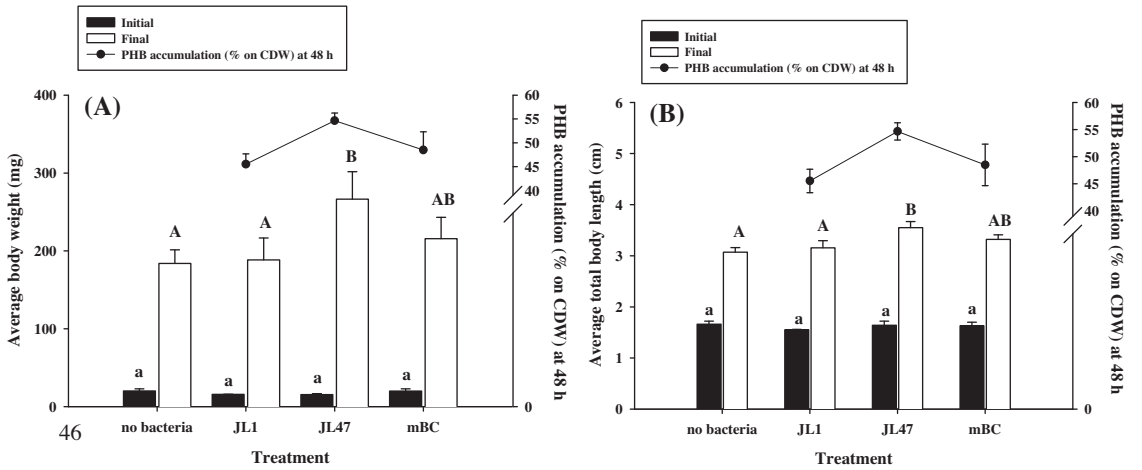

Fig. 3. Initial and final average body weight (A) and average body length (B) of *P. monodon* PL30 after 30 days of feeding with shrimp formulated diet added with and without different PHB-accumulating bacterial cultures (experiment 2). The PHB content of the bacterial cultures added to the feed is presented. Bars indicate means ± SEM ($n = 4$). Different letters indicate significant differences ($P < 0.05$) and small and capital letters denote different comparisons in a figure.

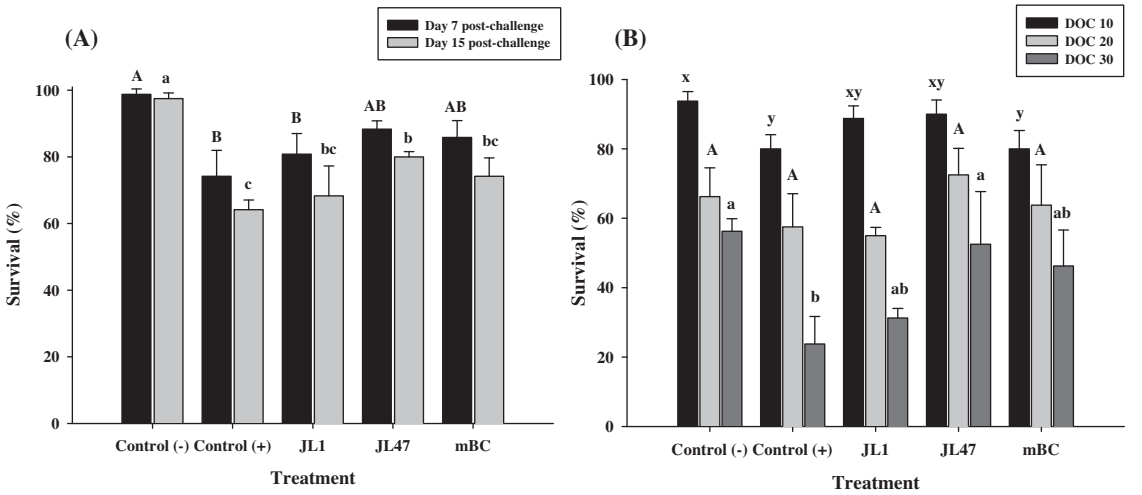

Fig. 4. (A) Survival of shrimp PL1 fed 30 days with diets supplemented with different PHB accumulating bacterial cultures and subsequently challenged for 24 h with pathogenic *Vibrio campbellii* LMG 21363 and a sub-lethal dose of ammonium chloride (75 mg L^{-1}) (experiment 1). (B) Survival of shrimp PL30 after daily addition of different PHB accumulating bacterial cultures and pathogenic *Vibrio campbellii* LMG 21363 at 10^6 CFU mL^{-1} of rearing water for 30 days during culture (experiment 3). Control (−) = non-challenged negative control, Control (+) = challenged positive control. Bars represent means ± SEM ($n = 4$). Different letters within a day indicate significant differences ($P < 0.05$). DOC = day of culture.

4. Discussion

The provision of PHB to *Artemia franciscana* in amorphous form (i.e. contained within a bacterial cell) seems to increase the efficiency as compared to the provision of PHB in crystalline form (i.e. extracted from the bacterial cell) (Halet et al., 2007). In this research, we were able to show that PHB accumulating *Bacillus* spp., a genus of bacteria often associated with strong probiotic effects, positively affected the culture efficiency and robustness of *P. monodon* postlarvae. The availability of PHB accumulating spore formers presents a major advance in PHB application as it allows for an efficient and long-term storage of the bacterial precursor of this beneficial compound.

The PHB accumulating bacterial cultures used in this study attained the highest PHB accumulation at 48 h of culture. The PHB content further decreased when bacteria reached their maximum growth (72 h). *Bacilli* are known to accumulate PHB during exponential growth to prepare for spore formation (Valappil et al., 2007). The stored PHB is then used as an energy source to fuel the actual sporulation process (Benoit et al., 1990). For this reason, the bacteria to be added in the diets of the experimental animals were harvested at 48 h, aiming to use them at their maximum PHB accumulation state.

Both shrimp PL1 and PL30 receiving PHB accumulating bacilli in the diet showed a higher survival as compared to the control shrimp. This gives a clear indication that the bacilli provided beneficial effects to the shrimp during

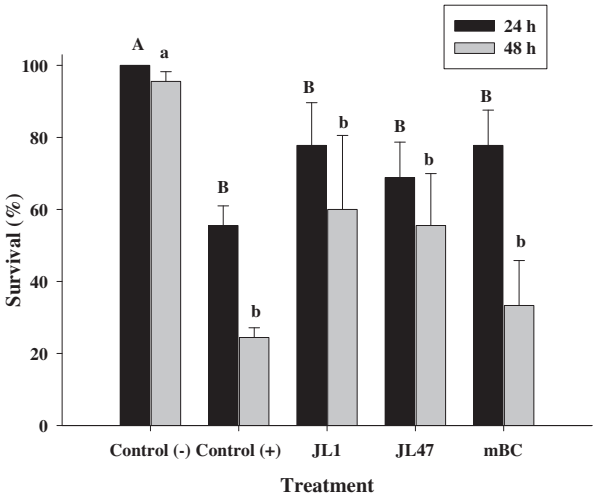

Fig. 5. Survival of shrimp PL30 fed 30 days with different PHB-accumulating bacterial cultures and subsequently exposed to a lethal dose of ammonium chloride (150 mg L^{-1}) (experiment 2). Bars represent means ± SEM ($n = 3$). Different letters indicate significant differences ($P < 0.05$) and small and capital letters denote different comparisons.

culture. In previous experiments, increased survival of crustaceans following crystalline PHB supplementation has been related to a decreased presence of opportunistic pathogens such as vibrios (Nhan et al., 2010). Potentially, a similar mechanism acted here. Growth was also better for the shrimp supplied of the PHB accumulating bacteria than those receiving the control diet. PHB has been reported to increase the growth performance of other aquatic animals as well (for example juvenile European sea bass in De Schryver et al. (2010) and fresh water prawn larvae in Nhan et al. (2010), although the exact mechanisms so far remain unknown. The improved growth of the shrimp PL30 in this study trended according the level of PHB in the diet. A significant correlation ($P = 0.01$) existed between the PHB dose in the diet and the increase in shrimp body weight as compared to the control ($r = 0.99$). These data suggest that the level of PHB in the diet was a main determinant for the growth performance of the shrimp. Several *Bacillus* spp., however, are known to produce exoenzymes, such as proteases, carbohydrolases and lipases, that can break down proteins, carbohydrates and lipids, respectively, and as such contribute to the digestion and absorption of feed in the gut (Arellano-Carbajal and Olmos-Soto, 2002; Wang, 2007; Ziaei-Nejad et al., 2006). The activity of the bacilli added to the feed may thus also have contributed to the growth performance of the shrimp. Further experimenting is required to determine the relative contribution of the PHB effect and a probiotic effect on the growth improvement that is achieved by supplying the PHB accumulating bacilli to the shrimp.

In addition to survival and growth, the application of the PHB accumulating bacteria affected the robustness of the shrimp as determined by several challenge tests. Two kinds of challenges were selected that are relevant for *P. monodon* larviculture: a pathogenic challenge test and an ammonia stress test. *V. campbellii* is an important pathogen for penaeid shrimp and among the *Penaeus* species *P. monodon* is the one most sensitive and susceptible to this pathogen (Hameed, 1995). In this study, shrimp were challenged with *V. campbellii* in two ways: (i) a single exposure to the pathogen in the rearing water in combination with a sub-lethal stressor (ammonium chloride) for 24 h; and (ii) a continuous exposure by daily addition of the pathogen in the rearing water for 30 days. In both variants, the survival was higher for the shrimp supplied of the PHB accumulating bacilli as compared to the shrimp from the challenged control, with a maximum attained for the shrimp supplied of the highest PHB accumulator (JL47). These observations correspond to what was found earlier on the application of PHB in challenge tests with the model organism *Artemia franciscana* (Defoirdt et al., 2007b; Halet et al., 2007). The suggested mode of action is that PHB is degraded intestinally resulting in a release of its monomer, 3-hydroxybutyrate. This compound can act antimicrobially by acidifying the cytoplasm of the pathogen. The pathogen has to redirect cellular energy to maintain homeostasis, resulting in a decreased virulence (Defoirdt et al., 2009). Alternatively, the PHB may have acted immunostimulatory toward the shrimp (Suguna et al., 2013). Similar as for the growth and survival results, however, it should also be taken into account that the different *Bacillus* cultures used in these experiments may have had probiotic activities contributing to the improved robustness of the shrimp during challenge. Additional tests will have to show if a probiotic effect of the bacilli also contributed to the observations. In this context, a set of preliminary in vitro experiments did not show a direct antagonistic effect of the bacilli toward the pathogen (data not shown).

Ammonia was applied as a second stressor as it is one of the most frequent stressors in high density larviculture of *P. monodon* (Chin and Chen, 1987). The results indicate that supplementation of the PHB accumulating bacteria may reduce the effects of ammonia stress, although there were no significant differences between the PHB treatments and the control. An explanation can be that PHB and its degradation products have earlier been found to act as an energy source for starved *Artemia* (Defoirdt et al., 2007b). The PHB can thus have contributed in the energy delivery to the shrimps increasing their strength to resist stress.

5. Conclusion

In conclusion, this study demonstrated the potential of newly isolated PHB accumulating bacilli as biocontrol agents and feed supplements in *P. monodon* larviculture. Further research is needed, however, to elucidate the relative contributions of the stored PHB and the probiotic activity of the bacilli in the survival, growth and robustness effects. This will allow optimizing the application of PHB accumulating *Bacillus* spp. for shrimp larviculture. In addition, it has to be noted that the method of culturing the PHB bacilli in LB medium for PHB accumulation may not be applicable to hatchery practices because of economical as well as practical reasons. A challenge lies in finding a cheap and easy method to overcome this

problem. An integrated technique consisting of a combination of *Artemia* culture to produce live food for early postlarval shrimp and the culture of the PHB accumulating bacilli may offer a solution. The strategy of growing bacteria on the waste metabolites released during *Artemia* hatching has been proposed earlier (Cam et al., 2009). By optimizing the conditions during *Artemia* hatching, such as C/N ratio in the medium, the PHB accumulating bacilli can most likely be triggered to accumulate high concentrations of PHB in situ.

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